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(71) Applicant: LIPOSOME TECHNOLOGY, INC. [US/US]; 1050 Hamilton Court, Menlo Park, CA 94025 (US).

(72) Inventor: YAU-YOUNG, Annie ; 1983 Annette Lane, Los Altos, CA 94022 (US).

(74) Agents: DEHLINGER, Peter, J. et al.; Ciotti & Murashige, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).

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(54) Title: CONTROLLED-RELEASE LIPOSOME DELIVERY SYSTEM

(57) Abstract

A method for selectively controlling the rate of release of a liposome-entrapped compound from an intramuscular or subcutaneous injection site. The method includes selecting the average size, amount, and lipid composition of liposomes injected into the site to produce a desired half life of release of the compound. A preferred composition used in practicing the invention includes an aqueous suspension of liposomes containing the compound in entrapped form, and having average particle sizes less than about 0.3 microns, and larger empty liposomes present in an amount effective to increase the half life of release of the compound from the injection site to a desired half life between about 1-14 days. Also disclosed is a stable liposome/calcitonin composition.

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CONTROLLED-RELEASE LIPOSOME DELIVERY SYSTEM

1. Field of the Invention

The present invention relates to a liposome-based system and method for delivering a pharmacologically active compound to the bloodstream at a controlled rate.

2. References

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3. Background

Liposome delivery systems have been proposed for a variety of pharmacologically active compounds, such as drugs and peptide hormones. For compounds which are administered parenterally, liposomes have the potential of providing controlled "depot" release over an extended time period, and of reducing toxic side effects, by limiting the plasma peak level of the free compound in the bloodstream. These combined advantages allow the compound to be administered less frequently, and at a higher dose level, thereby increasing the convenience of the therapy.

One route of liposome administration which has been widely proposed for parenteral administration is

intravenous (IV) injection. Liposomes administered by this route are generally cleared by the reticuloendothelial system (RES), and as a consequence, the liposomes tend to concentrate in organs, such as liver, spleen, and lung, which are rich in RES cells. The ability to direct liposomes somewhat specifically to RES-rich tissue is advantageous, for example, in treating diseases of the liver, spleen, or lungs. This approach is described, for example, in U.S. Patent Application for "Liposome/Anthraquinone Drug Composition and Method," Serial No. 806,084, filed 6 December 1985, which discloses an improved therapy for treating hepatic tumors by liposome-entrapped doxorubicin. Often however, when the compound to be administered is intended for a site other than a RES-rich tissue, IV administration may be of limited use, particularly if long-term release into the bloodstream is needed.

Intramuscular (IM) or subcutaneous (SQ) administration of liposome-entrapped compounds have also been proposed. This approach has the advantage that, as long as the liposomes are contained at the site of injection, rapid uptake and clearance by the RES cannot occur. The liposomes immobilized at the site of injection can then release the entrapped compound into the bloodstream over an extended period. By way of example, U.K. Patent Application No. 2,050,287 describes a liposome system which is intended for slow release of insulin from an SQ injection site. More recently, a system for releasing liposome-encapsulated calcitonin from an IM site of administration has been proposed (Fukunaga).

It would be desirable, in an IM or SQ delivery system of the type just mentioned, to be able to control the rate of release of the liposome-entrapped compound

from the site of injection. Prior art attempts to understand and control the variables which affect drug-release rates from the injection site have had only limited success, however. One source of difficulty in these studies is the problem of separating factors which affect the rate of breakdown and/or release of liposomes at the site of injection from those which affect release of the entrapped compound from liposomes. For example, if a liposome-permeable drug is studied, the rate of drug release into the bloodstream may be largely independent of liposome stability at the site of injection.

One study which has been reported to date examined the effect of several liposome variables on release of liposome-entrapped inulin from an IM injection site (Jackson). The general conclusions were that (a) smaller liposomes (0.15-0.7 microns) are taken up more rapidly by the lymphatics than larger liposomes (0.3-2.0 microns), and (b) decreasing the total amount of liposomes injected resulted in slower absorption of liposomes from the injection site. Neither of these conclusions predict how one would control the rate of release of a liposome-impermeable agent, such as peptide, from a liposomes administered to an IM site. Nor is it clear how larger liposomes could be prepared in a sterile form for use in human therapy.

4. Summary of the Invention

It is therefore a general object of the invention to provide a system and method for controlling in a selected manner, the rate of release of a liposome-impermeable compound from an IM or SQ site.

Another object of the invention is to provide a such a system which can be readily prepared in sterile form suitable for IM or SQ administration in humans.

Still another object of the invention is to provide a stable liposome/peptide hormone composition for use in parenteral hormone delivery at a controlled release rate.

The invention includes a method for selectively increasing the rate of release of a liposome-impermeable compound into the bloodstream. The compound is administered by forming a suspension of liposomes which contain the compound in entrapped form, and injecting the suspension into an IM or SQ site. The rate of release of the agent from the site is increased selectively by increasing the average size of the liposomes and the total amount of liposome lipid injected into the site. The encapsulated agent may be a peptide, such as insulin, growth hormone, or calcitonin, interferon, or interleukin-2, which is advantageously released into the bloodstream at a controlled rate over a several day period.

The rate of release of the liposome-encapsulated agent can also be controlled by changes in the lipid composition of the liposomes. Negatively charged phospholipids, such as phosphatidylglycerol (PG), act to decrease the release rate, and cholesterol, to increase the rate.

In one embodiment, the liposomes are prepared by first forming a suspension of small, compound-encapsulating liposomes, removing the non-encapsulated compound from the suspension, filter sterilizing the suspension, then adding sterilely prepared empty liposomes to the suspension until the desired liposome average size and concentration are

reached. The small liposomes are preferably less than about 0.3 microns in size, and the empty liposomes, 0.5 micron or more.

The invention also includes a liposome composition for administering a liposome-impermeable compound to the bloodstream from an IM or SQ site of injection. The composition is composed of an aqueous suspension of liposomes containing the substance in entrapped form, and having average particle sizes less than about 0.3 microns, and a quantity of empty liposomes, in an amount effective to increase the half life of clearance of the substance from such injection site to a desired half life between about 1-14 days. The size, lipid composition, and relative quantity of empty liposomes are selected to produce a desired rate of release of the encapsulated agent from the site of injection.

In still another aspect, the invention includes a liposome/calcitonin (CT) composition comprising a sterile, aqueous suspension of liposomes containing at least about 0.2 mole percent α-tocopherol, and CT entrapped in the liposomes at a concentration within the encapsulation space of the liposomes of at least about 0.1-1 mg/ml. The composition may be further stabilized by the presence of ferrioxamine, in molar excess of the amount of ferric iron in the suspension.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 is a flow diagram of a processing method used in preparing a liposome composition containing both empty and peptide-encapsulating lipids;

Figure 2 shows the kinetics of release of a liposome tracer lipid from the site of an IM injection (circles) and the accumulation of tracer (triangles) excreted from the injected animal;

Figure 3 is a semi-log plot of the tracer release data from Figure 2 (dotted line) and analogous tracer release data from liposome composition with smaller liposome sizes (solid line);

Figure 4 shows the kinetics of release of encapsulated radioactive calcitonin (CT) from the site of an IM injection (circles), the accumulation of hormone (triangles) excreted from the animal, and release of free CT from the site of injection (squares); and

Figure 5 is a semi-log plot of CT release data from Figure 4.

Detailed Description of the Invention

1. Preparing the Liposome Composition

A. Lipid Components

The liposomes in the composition are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by considerations of (a) desired liposome size and ease of liposome sizing, and (b) lipid and hormone release rates from the site of liposome injection.

Typically, the major lipid component in the liposomes is phosphatidylcholine (PC). PCs having a variety of acyl chain groups of varying chain length and degree of saturation are available, or may be isolated or synthesized by well-known techniques. In general, less saturated PCs are more easily sized, particularly when the liposomes must be sized below about 0.3 microns, for purposes of filter sterilization. Methods used in sizing and filter-sterilizing liposomes are discussed below. The acyl chain composition of the phospholipid may also affect the rate of clearance of liposome lipids and entrapped compound from the site injection, although acyl-chain saturation effects appear to have less of an effect on drug release rates than when liposomes are administered intravenously. One preferred PC is egg PC (EPC) which is derived from egg lipids, and contains a mixture of both saturated and unsaturated acyl chain groups.

Experiments conducted in support of the present invention, and reported in Examples III and V below, show that negatively charged phospholipids significantly increase the rate of clearance of lipid and entrapped compound from the site of an IM injection, when compared with liposomes formed from PC alone or PC/cholesterol mixtures. Although the studies involved liposomes formulated with selected mole ratios of PG, other negatively charged phospholipids, such as phosphatidylserine (PS), and phosphatidylinositol (PI), could be used. The PG effect observed appears to be related, in part, to the ability of charged lipids to prevent spontaneous liposome aggregation. Size measurements on liposomes after sizing by extrusion through a 1 micron pore size polycarbonate membrane, reported in Example IV, show that PG containing

liposomes have stable sizes of about 1 micron, whereas liposomes containing only PC have particle sizes between about 3-5 microns. As will be seen below, and according to one important feature of the method of the invention, larger liposome sizes show longer drug release times at an IM site of injection.

Evidence presented in Examples IV and V indicates that negatively charged phospholipids may also increase in situ lipid and drug release by a mechanism unrelated to liposome size. Briefly, liposomes composed of pure PG (plus a small amount of α-tocopherol) showed more rapid clearance of lipid tracer and a radiolabeled encapsulated peptide than similar-sized liposomes containing only 5 or 10 mole percent PG. Negatively charged phospholipids having a variety of acyl chain components are available. One preferred lipid is egg PG (EPG) which contains a mixture of saturated and unsaturated acyl chain moieties.

The effect of added cholesterol on drug and lipid release from an IM site of injection was also examined. In general cholesterol is known to increase liposome stability, and therefore might be expected to increase the time required for clearance of lipid and entrapped components from an IM site. As reported in Example V, liposomes containing PC and cholesterol, in a mole ratio of about 6:4, showed approximately 20% longer release times of encapsulated peptide than pure PC liposomes. However, addition of cholesterol to PG-containing liposomes produced very little change in the rate of release of either lipid or encapsulated peptide from the site of injection.

B. Protective Agents

It is well known that the lipid components of liposomes promote peroxidative and free radical reactions which cause progressive degradation of the liposomes. This problem has been discussed at length in the above-mentioned patent application for "Anthraquinone/Liposome Composition and Method". Briefly, that application reported that lipid peroxidative and free radical damage degrades both lipid and entrapped drug components in a liposome/drug composition. The extent of free radical damage to lipid and drug components was reduced significantly when a lipophilic free radical quencher, such as α-tocopherol (α-T) was included in the vesicle-forming lipids. Interestingly, a significantly greater reduction in lipid damage and drug modification was observed when the liposome/drug composition was formulated in the presence of both α-T and a water-soluble, iron-specific chelator, such as ferrioxamine. Since ferrioxamine can complex tightly to ferric iron at six coordination sites, it is likely that the compound act by inhibiting iron-catalyzed peroxidation in the aqueous phase of the liposome suspension. The effectiveness of the two protective agents together suggests that both iron-catalyzed peroxidative reactions occurring in the aqueous phase, and free radical reactions being propagated in the lipid phase are important contributors to lipid peroxidative damage.

The lipophilic free radical scavenger used in the composition is preferably α-T, or a pharmacologically acceptable analog or ester thereof, such as α-T succinate. Other suitable free radical scavengers include butylated hydroxytoluene (BHT), propyl gallate (Augustin), and their pharmacologically

acceptable salts and analogs. Additional lipophilic free radical quenchers which are acceptable for parenteral administration in humans, at an effective level in liposomes, may also be used. The free radical quencher is typically included in the lipid components used in preparing the liposomes, according to conventional procedures. Preferred concentrations of the protective compound are between about 0.2 and 2 mole percent of the total lipid components making up the liposomes; however, higher levels of the compound, particularly α-T or its succinate analog, are compatible with liposome stability and are pharmacologically acceptable.

The water soluble iron-specific chelating agent is selected from the class of natural and synthetic trihydroxamic acids and characterized by a very high binding constant for ferric iron (on the order of 10^{30}) and a relatively low binding constant for 2-valence cations, such as calcium and magnesium. A variety of trihydroxamic acids of natural origin are known, including compounds in the ferrichrome class, such as ferrichrome, ferrichrome A, and albomycin; compounds in the ferrioxamine class, including the ferrioxamines and ferriomycines; and compounds in the fusaramine class.

One preferred chelator is ferrioxamine B, also known variously as ferrioxamine, desferrioxamine, desferrioxamine B, and Desferal™. This compound shows exceptional iron binding affinity and has been proven safe for parenteral use in humans in treating iron-storage disease and iron-poisoning.

The chelating agent is present in the composition at a concentration which is in molar excess of the ferric iron in the liposome suspension.

Typically, aqueous media used in liposome preparation contains at least about 1-2 μM ferric iron, and may contain up to 100 μM or more ferric iron. For aqueous medium containing up to about 20 μM iron, concentrations of chelating agent of about 50 μM are preferred.

The chelating agent is preferably added to vesicle-forming lipids at the time of liposome formation, so that the lipids are protected against drug-promoted lipid oxidation damage during liposome preparation. Methods for preparing liposomes by addition of an aqueous solution of chelating agent are described below. Here it is noted only that the liposome suspension formed by this method contains chelating agent both in the bulk aqueous phase and in the encapsulated form, i.e., within the aqueous internal liposome region. Alternatively the chelating agent may be included in the suspension after liposome formation.

C. Entrapped Compound

The compound entrapped in the liposomes is a liposome-impermeable drug or peptide whose rate of diffusion out of liposomes is not significantly greater than the rate of breakdown of liposomes at an IM site of injection. The agent may be either a lipophilic drug or hormone whose oil/water partitioning strongly favors the liposome bilayer phase, or a water-soluble drug or peptide which is capable of diffusing across the liposomal bilayer slowly, if at all. Specifically excluded from the invention are water-soluble drugs which can freely diffuse out of liposomes with a half life of less than a few hours.

Peptide hormones and immunological activators are one important class of compounds for use in the

invention. Representative peptide hormones include insulin, growth hormone, and calcitonin (CT), which regulates calcium blood levels. Interferon and interleukin-2 are representative of immunological activators. THe present invention allows the selected peptide compound to be released into the bloodstream at a slow, controlled rate over a several hour to several day period, thus avoiding the large fluctuations in blood peptide levels that are characteristic of free hormone administration.

In addition, and in accordance with one of the discoveries of the present invention, the carrier liposome may significantly enhance the stability of the peptide on storage, when the peptide is present at a relatively low concentration. The study reported in Example II shows that the stability of CT in free form is enhanced severalfold when the CT concentration is raised from 0.010 to 1.0 mg/ml. CT which is encapsulated in liposomes at a relatively high concentration -- e.g., 1 mg/ml -- but present at a relatively low bulk phase concentration -- e.g., 0.010 mg/ml -- shows the same stability effect seen at high concentrations of the free hormone. Thus, the invention allows peptide hormones such as CT which are more stable at high concentration to be stored and delivered in a relatively dilute form in which the high-concentration microenvironment of the hormone promotes good stability on storage. The preparation of liposomes which encapsulate water-soluble peptides, such as CT, at a selected internal volume concentration are considered below.

Steroid hormones and anti-inflammatory agents are another important class of compounds which are useful in the present invention. Representative

steroids include hydrocortisone, estradiol, and testosterone. Liposomes containing entrapped steroids are readily formulated by including the compounds in vesicle-forming lipids. The rate of release of the steroids from the site of IM or SQ injections would be controlled by the partition function of the drug, as well as by liposome stability in and migration from the site of injection.

Other types of compound which are suitable for slow release liposome delivery from an IM or SQ site include antibiotics, such as as amphoteracin B, immunosuppressives, such as cyclosporin, and anti-tumor agents, such as doxorubicin. Co-owned patent application for "Amphoteracin B/Liposome Composition", Serial No. 781,395, filed 27 September 1985, describes a novel liposome composition containing a high molar ratio of amphoteracin B (up to 20 mole percent of the total lipid) in a form which is compatible with stable liposome sizes. One feature of the present invention which is particularly advantageous for antibiotic or anti-tumor drug delivery is the ability to select the site of drug release and highest drug concentration.

D. Liposome Preparation

A variety of methods are available for preparing liposomes, and these have been reviewed at length by Szoka (1980). One preferred method produces multilamellar vesicles (MLVs) of heterogeneous sizes. In this method, vesicle-forming lipids, including a lipophilic free radical protecting agent, and if suitable, a lipophilic drug compound, are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable

solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with hydration medium and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting MLVs can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions.

In forming liposomes with an encapsulated water-soluble compound, such as a peptide hormone, the compounds is dissolved in the hydration medium at a concentration which is desired in the interior volume of the liposomes in the final liposome suspension. Thus, for example, to form liposomes encapsulating CT at an interior space concentration of about 1 mg/ml, the hydration medium would contain 1 mg/ml CT. The hydration medium may also be prepared to include an iron-specific chelater, at a preferred concentration of between about 10-50 mM. Example I describes a method of producing a suspension of MLVs encapsulating CT.

Another advantageous method of producing liposomes is the reverse-phase evaporation method described by Szoka (1978). Here a solution of vesicle-forming lipids in an organic solvent or solvent system is added to an aqueous solution of the material to be encapsulated, at relative volume amounts which are compatible with a water-in-oil emulsion. The mixture is then emulsified, and the organic phase removed to produce a reverse-phase lipid gel composed of lipid monolayer structures encapsulating aqueous droplets. This gel, when resuspended in an aqueous solution, forms a suspension of relatively large oligolamellar vesicles, commonly referred to as reverse-evaporation vesicles (REVs). The method produces high encapsulation

efficiencies, typically between about 30-40% of the total water-soluble material added, and is thus useful for encapsulating expensive drug or peptide compounds, such as peptide hormones.

Regardless of the method used, liposome preparation is preferably carried out under conditions which lead to a sterile liposome suspension. This is accomplished by employing conventional sterile techniques throughout the procedure.

E. Liposome Sizing

Even though initial liposome preparation may be done under sterile conditions, it is generally necessary to treat the preparation to remove free drug, and such treatment generally involves methods which are difficult to carry out aseptically. Therefore, a final sterilization may be required before the liposomes can be used for parenteral injection.

The method of choice for liposome sterilization, and the only method available for sterilizing liposomes containing heat-sensitive encapsulated material, is by filtration through a conventional depth filter, typically a 0.22 micron filter. This method can be carried out on a practical, high through-put basis only if the liposomes have first been sized down to about 0.2-0.3 microns or less.

Several techniques are available for reducing liposome to this size range. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, MLVs are

recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination.

Extrusion of liposome through a small-pore polycarbonate membrane is an effective method for reducing liposome sizes down to a relatively well-defined size distribution (Szoka 1978). Typically, the suspension is cycled through the membrane several times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

F. Removing Free Drug

Even under the most efficient encapsulation methods, such as the REV method mentioned above, the maximum encapsulation efficiency for a water-soluble compound is about 50%, so that the initial liposome suspension will contain 50% or more of the compound in free (non-entrapped) form. The amount of non-encapsulated material is still higher in a liposome suspension formed by the above MLV method, where encapsulation efficiencies between about 10-20% are typical. The studies reported in Example V show that a free (non-encapsulated) peptide is cleared from the site of an IM injection in less than an hour, as compared with several hours to several days for liposomes encapsulated compound. Therefore, in order to minimize the effect of rapid drug release from the site of injection, it is important to remove free drug which may be present in the injected composition.

Several methods are available for removing non-entrapped compound from a liposome suspension. In one simple method, the liposomes in the suspension are pelleted by high-speed centrifugation, leaving free compound and very small liposomes in the supernatant. Another method involves concentrating the suspension by ultrafiltration, the resuspending the concentrated liposomes in a drug-free replacement medium. Alternatively, gel filtration can be used to separate larger liposome particles from solute molecules.

Yet another approach for removing free compound utilizes ion-exchange, molecular sieve, or affinity chromatography. Here the liposome suspension is passed through a column containing a resin capable of binding the compound in free, but not entrapped, form, or a support having attached binding molecules, such as antibodies, which bind specifically to the non-encapsulated compound. The approach may also be effective in removing free pyrogens, with proper selection of resin(s).

Following treatment to remove free drug, the liposome suspension is brought to a desired concentration for use in IM or SQ administration. This may involve resuspending the liposomes in a suitable volume of injection medium, where the liposomes have been concentrated, for example by centrifugation or ultrafiltration, or concentrating the suspension, where the drug removal step has increased total suspension volume. The suspension is then sterilized by filtration as described above.

G. Liposome Processing

Figure 1 is a flow diagram of a liposome-processing scheme suitable for preparing the liposome

composition of the invention. THe flow scheme at the left in the figure shows steps in forming a sterile suspension of liposomes which have an entrapped compound. THese steps, which have been detailed above, involve first preparaing a suspension of liposomes containing the compound at a selected entrapped concentration. The suspension is then sized down to 0.2-0.3 or less, to allow for eventual sterile filtration. After removing non-entrapped drug, the material is brought to a desired lipid concentration and sterilized by filtration.

The righthand column in Figure 1 illustrates parallel steps used in preparing empty liposomes. The rationale for preparing empty liposomes is based on the discovery herein that the release rate of liposome-encapsulated material from an IM or SQ injection site can be selectively varied by changing (a) the lipid composition, (b) average liposome size, or (c) total lipid amount of the injected liposomes. The relationship between these parameters and compound release rate will be detailed in Section II below, and in Example III-VI. At this point, it is sufficient to note that these effects are achieved even when a large portion of the liposomes injected into the site do not contain entrapped compound. That is, the effect of lipid composition, size, and amount on compound release rate appear to depend on liposome/liposome interactions which affect empty and compound-containing liposomes in the same way.

The advantage of empty liposomes, as a method of varying lipid composition, size, and amount, is that they can be prepared in sterile form without the need of a final sterile filtration step. It will be recalled that the sterility of a liposome preparation is

compromised primarily because of the need for remove free compound from the suspension. By forming liposomes in the absence of the compound, or in the presence of an amount which does not produce an appreciable amount of free compound, the final sterile filtration step can be avoided. An important advantage here is that since the empty liposomes do not have to be sized down for filtration, relatively large liposomes can be added, allowing for greater size-related effects in compound-release rate to be achieved.

Considering the scheme shown at the right in Figure 1, the empty liposomes may be treated, if desired, to remove smaller liposomes. This can be done, for example, by allowing larger liposomes to settle in a vessel, and aseptically removing non-settled material. Alternatively, the liposomes may be added directly to the filter-sterilized liposomes, without further treatment, to produce a final composition having a selected average lipid composition, size, and concentration, or the liposomes may be sized, such as by extrusion through a 1 micron polycarbonate membrane under sterile conditions, before addition to the filter-sterilized liposomes.

The processing method described above thus allows for the preparation of a sterile liposome composition having (a) a selected concentration of entrapped compound, (b) little or no free compound, and (c) an average liposome size, concentration and composition which allows the entrapped compound to be released from an injection site at a selected release rate.

2. Properties of the Composition

A. Stability

One advantageous feature of the present invention is the increased stability of a pharmacological compound, such as a peptide hormone, which is achieved with liposome encapsulation. In particular, where the compound is more stable in a concentrated form, such as has been found for CT, liposome encapsulation allows the compound to be stored in stable form in a suspension in which the compound has a high localized concentration, but at relatively low overall concentration. For example, the study reported in Example II shows that free CT is substantially more stable at 1 mg/ml than at 0.01 mg/ml, even in the presence of a carrier protein. When the peptide is encapsulated in liposomes at an internal-volume concentration of 1 mg/ml, but at at suspension concentration of 0.01 mg/ml, the peptide shows the stability characteristics of the high concentration of CT.

The stability of the liposomes is enhanced by including a lipophilic free radical quencher, such as α-T, at a mole ratio of at least about 0.2. Further, composition stability may be achieved by addition of an iron-specific chelator, such as ferrioxamine, in molar excess of the amount of free ferric iron in the liposome suspension. The CT/liposomes can be sized, treated to remove free CT, and sterile filtered, as above. The resulting composition includes (a) a sterile, aqueous suspension of liposomes containing at least about 0.2 mole percent α-T, and (b) calcitonin entrapped in the liposomes at a concentration of at least about 1 mg/ml.

The concentration of free CT is preferably less than about 10 mole percent of the encapsulated peptide.

The discovery that liposome encapsulation allows a peptide hormone, such as CT, to be stored in stable, dilute form applies to a variety of other peptides and proteins. For example, the approach could be used to store enzymes in stable, dilute form.

B. Release Characteristics of Lipids and Encapsulated Compound

The clearance rates of lipids and an encapsulated compound from an IM site of injection have been examined, as reported in Examples III-VI. The purpose of the studies was twofold: (1) to determine the relationship between lipid and peptide clearance from an injection site, and (2) to examine the liposome variables that affect peptide release rate. although the studies were performed using CT as a model peptide compound, it will be recognized that the findings are applicable to any water-soluble compound that would require liposome breakdown for its release into the bloodstream.

To follow the rate of clearance of liposome lipids from an IM injection site, liposomes containing ^{125}I-labeled phosphatidylethanolamine (^{125}I-PE) as a tracer lipid were injected into the limbs of laboratory animals. The disposition of the tracer lipid was measured at a minimum of eight time points over at least a 70-hour test period. The loss of tracer from the sites of injection (circles) and accumulation of the excreted tracer (triangles) are seen in Figure 2. The data on tracer clearance from the site of injection, when plotted as a semi-log function, gives a linear plot, such as seen in Figure 3, from which the lipid

clearance half life can be calculated. Details of the method are given in Example III.

The clearance of radiolabeled CT from liposomes injected IM in lab animals was similarly followed. Figure 4 shows plots of the loss of tracer from the sites of injection (circles) and accumulation of excreted tracer (triangles) from a typical experiment. A semi-log plot of the CT clearance, shown in Figure 5, was used to calculate clearance half life from the site of injection. The linear relationship between the log of CT retained and time indicates that lipid is cleared from the site of injection with first order kinetics. Also shown in the figure is the rapid clearance of free CT from the sites of injection into the bloodstream.

The effect of liposome size on lipid and CT clearance rates is apparent from the data in Example IV comparing liposomes with sizes ranging from 0.2 microns to about 5 microns, with larger liposomes showing 60-100% longer lipid clearance rates. The data in Example VI show that the clearance of lipid tracer from small liposomes (about 0.2 microns) can be increased by addition to the injected liposomes of larger unlabeled liposomes (about 1 micron). The latter results indicate that lipid clearance is governed by bulk effects related to average liposome sizes, and forms the basis, according to one aspect of the invention, of controlling release characteristics of smaller liposomes by the addition of larger, empty ones.

The effect of liposome dose on lipid clearance is also reported in Example IV. As discussed therein, lipid clearance half life can be increased more than twofold with increased lipid dose, independent of lipid size or composition. The same twofold increase in clearance rate with increased dose is seen for

encapsulated CT (Example V). Interestingly, a comparison of lipid and CT release rates from liposomes having the same size and dose properties (Example V) shows that CT clearance from the site of injection is about twice as fast as that of the corresponding lipid tracer. This finding suggests that liposomes are destabilized and release their encapsulated contents predominantly at the site of injection, with lipid clearance from the site being handled by a different, slower mechanism.

The effect of lipid composition on lipid and CT clearance from an IM site was also examined. As discussed above, and reported in Examples IV and V, addition to the liposomes of a negatively charged phospholipid, such as PG, significantly increases the rate of clearance of lipid and encapsulated CT from an IM site. As noted in Example IV, the PG effect may be related in part to the reduced liposome aggregation which is seen in PG-containing liposomes.

Cholesterol, at a mole ratio of about 40%, had little effect on lipid clearance from PG-containing liposomes, but showed a significant stabilizing effect -- that is, longer clearance half life -- on CT release from neutral, PC liposomes. CT was cleared about twice as rapidly as lipid tracer from liposomes having the same size and lipid composition properties, confirming that liposome lipid is cleared from the injection site by a different, slower mechanism than that acting on the encapsulated compound.

The results above show that release of an encapsulated compound from liposomes at an injection site can be controlled selectively by changes in liposome size, dose, and lipid composition. specifically, in practicing the method of the invention,

the rate of release of an encapsulated compound from an injection site is controlled according to an average size and total amount of liposome injected into the site. In one aspect of the invention, average size and lipid amount are selectively increased by adding larger, empty liposomes to smaller, filter sterilized liposome encapsulating the compound of interest.

Greater release time can also be achieved, according to the invention, by adding cholesterol to either or both of the compound-containing or empty liposomes. To achieve faster release of the encapsulated compound, the liposomes can be formulated to include progressively more negatively charged lipid, such as PG.

3. Therapeutic Uses

The liposome composition of the invention is useful for administering a variety of liposome-impermeable compounds parenterally. One important application is for use in administering a peptide hormone or immunostimulator to bloodstream in a controlled fashion over a several day period. The composition allows the half life of peptide release to be selectively varied, to provide release for a selected period of up to several days. The peptide can then be given less often and without the sharp fluctuations seen when free peptide injections are used. Further, a greater degree of control can be achieved than with liposome formulations which have been proposed heretofore.

Insulin and CT are examples of peptides which are now routinely administered in free form. Both of these peptides are readily incorporated into the liposome composition of the invention, and both can be

delivered over a several day period by IM injection of the composition. The rate of hormone delivery is controlled, according to the method of the invention, by use of selected average liposome size, amount, and composition. For some peptides, such as CT, an added benefit of the liposome composition is the increased stability which is achieved, allowing the material to be stored in relatively dilute form over an extended period.

The following examples describe particular embodiments of making and using the invention, but are in no way intended to limit the invention.

Example I

Preparation of CT/MLVs

Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol were supplied by Avanti Lipid (Birmingham, AL). The lipids were judged about 99% pure by thin-layer chromatography. Cholesterol (CH) and α-tocopherol (α-T) were obtained from NuChek Prep, Inc. (Elysian, MN) and Sigma Chemical Co. (St. Louis, MO), respectively, both at a purity of about 99% or greater. Salmon calcitonin (sCT) was a gift of Armour Pharmaceuticals, Kankakee, IL, and sCT was ^{125}T-radiolabeled with ^{125}I by the chloramine T method (McFarland).

Multilamellar vesicles (MLVs) containing one of the lipid compositions A-E indicated in Table I were prepared. The molar ratios of the lipid components in the four vesicle preparations are shown in Table I. The values in the table indicate the μmolar ratios of each lipid component that were used in forming the various vesicle preparations. For studies to measure the rate of clearance of liposome lipid from an intramuscular

site of injection, the vesicle-forming lipids also included a radioactive iodinated derivative of phosphatidylethanol- amine (^{125}I-PE) tracer. To form the tracer, the p-hydroxybenzamide of egg phosphatidylethanolamine (EPE) was synthesized as described by Abra, and this compound was iodinated with ^{125}I, as described by Greenwood. The specific activity of the stock was 3.85×10^6 cpm per nmole PE. The iodinated lipid was incorporated at 1×10^5 cpm per injection in the range of 0.2 to 10 μmole total lipid.

TABLE I

Composition	EPC	EPG	CH	α-T
A	99			1
B	94	5		1
C	49	5	40	1
D		99		1
E	59		40	1

To form MLVs, the lipid components in chloroform stock solution were mixed in a tube or round bottom flask. The chloroform was removed by rotaevaporation and the lipid mixture was dissolved in tert-butanol. The butanol solution was then frozen in dry-ice/acetone and lyophilized overnight. The dry lipids were hydrated in several ml of phosphate-buffered saline (PBS), pH 7.4 For the studies involving CT/MLVs, the hydration buffer contained 0.2-5 mg/ml of ^{125}I-sCT, and sCT.

The lipid film was hydrated with vortexing for about 15 minutes at room temperature to form an MLV suspension having heterogeneous sizes ranging from about 0.2 to 10 microns. The vesicle preparations were sized

by extrusion through a polycarbonate membrane having selected pore sizes. The entire preparation was extruded through a 1.0 micron polycarbonate membrane, producing vesicles which have an initial vesicle size (before aggregation) of about 1 micron. In forming smaller-size vesicles, the sized vesicles were further extruded successively through 0.4 and 0.2 micron pore size membranes, to produce vesicles with sizes initially in the 0.2 micron size range.

MLVs containing encapsulated sCT were freed of non-liposome-associated free sCT by three washes with PBS. The formulations were tested for pyrogen by the Limulus Amoebic Lysate assay (Haemachem, Inc., St. Louis, MO).

Example II

Stability of Liposome Encapsulated sCT

Composition B MLVs containing 1.0 mg/ml encapsulated sCT were prepared as in Example I. The vesicles were extruded successively through 1.0 and 0.4 polycarbonate filters and washed three times in PBS to remove free sCT. The liposomes were diluted to final concentration of 0.010 mg/ml sCT, and sterilized by passage through a standard sterile 0.45 ml depth filter, and aliquoted into sterile Nunc vials. Sterilized solutions of free sCT containing either 0.010 or 1.0 mg/ml in PBS with 0.5% BSA were also aliquoted into the vials. The liposome-encapsulated and free sCT preparations are referred to in Table II as L-sCT and F-sCT, respectively.

The stability of the F-sCT and L-sCT preparations was tested after incubating the vials at either 4°C, room temperature (about 24°C), and 37°C, and assaying the biological activity of the sCT after a

given period of incubation. The samples were lysed in 0.5% Triton-X, obtained from Sigma (St. Louis, MO), and diluted to 40 and 120 mU per ml of PBS containing 0.5% BSA. An aliquot of frozen standard F-sCT was similarly treated.

Each sample was tested in eight rats, with four rats receiving 10 mU of sCT (0.25 ml of the 40 mU/ml solution), and four rats receiving 30 mU (0.25 ml of the 120 mU/ml solution), administered subcutaneously. Control rats received 0.25 ml of PBS/BSA. At exactly sixty minutes after the injection, blood samples were drawn from each rat. The blood samples were allowed to clot, and serum samples collected by centrifugation. The calcium levels in the serum were determined by a dye binding assay, using a kit supplied by Sigma Chemical Co. (St. Louis, MO). The hypocalcemic activity of the samples, 60 minutes after injection, was determined from a standard curve. The results, expressed as the average of four rats for each data value, are given in Table II.

TABLE II

No.	Sample	[sCT] (mg/ml)	Temp. (°C)	Time (days)	Activity (%)
1	F-sCT	0.01	4	128	27
2	F-sCT	1.0	4	128	100
3	L-sCT	0.01	4	128	100
4	L-sCT	0.01	4	215	100
5	F-sCT	0.01	RT	43	40
6	F-sCT	1.0	RT	43	100
7	L-sCT	0.01	RT	43	100
8	F-sCT	1.0	RT	98	61
9	L-sCT	0.01	RT	98	62
10	F-sCT	1.0	37	7	62
11	L-sCT	0.01	37	7	74

The first three rows compare the stability of dilute and concentrated solutions of F-sCT and a dilute solution of L-sCT over a 128-day period at 4°C. As seen, dilute F-sCT, but not L-sCT, loses most of its activity during this period. The liposome stabilizing effect is presumably due to the locally high concentration of sCT (about 1 mg/ml) within the liposome encapsulation space. As seen in row 4, encapsulated sCT in a dilute solution is stable for at least 215 days.

A similar liposome-protective effect is seen with sCT incubated at room temperature. Over a 43-day incubation period, a dilute solution of F-sCT has lost about 60% of its activity, but the concentrated F-sCT and dilute L-sCT preparations showed no activity loss. That the latter two preparations have about the same stability is seen from the data for 98-day incubation at room temperature, and that for 7-day incubation at 37°C.

Example III

Lipid Clearance From the Site of IM Injection

The study reported here examines the rate of clearance of liposome lipids, as measured by a radioactive lipid tracer, from the site of intramuscular (IM) injection in laboratory rats. The animals were injected with MLVs having one of the lipid compositions A-D from Example I, at a selected dose of 0.2, 2, or 10 μmole lipid.

Male Sprague-Dawley rats weighing 95-110 g were lightly anesthetized with ether, and 20 μl containing 0.2 μmole lipid was injected into the forelimbs or 100 μl containing 2 to 10 μmole lipid was injected into the hindlimbs of each rat. Sixteen rats were used in each experiment. Two of the rats were placed in metabolic cages to monitor the excretion of radiolabel

in urine and feces. Eight time points were taken for each experiment over a test period of 70 hours to 6 days. The eight time points, which are seen in Figures 1 and 2 for a typical experiment, were selected so that the half life of lipid clearance from the IM site can be determined by a semi-log plot of radioactivity remaining versus time after injection (Figure 2). At each time point, two rats were anesthetized for blood sampling, in which 2 ml of blood were withdrawn and counted for ^{125}I radioactivity. The rats were then killed by asphixiation in a CO_2 chamber, and the injected limbs were dissected and counted for radioactivity. Urine and feces were collected from the two animals in the metabolic cages throughout the experiment. These two animals were also sacrificed, serving as the last time point, and were also dissected for their thyroid, lungs, heart, liver, spleen, stomach, intestines, and genitals for counting. The remaining carcass was digested prior to counting. Radioactivity levels were measured to determine the disposition of residual lipid tracer in the animals at the end of the experimental period.

The results of a typical experiment are shown in Figure 2. The upper curve (circles) represents radioactivity retained at the site of injection (the limb sites), expressed in percent of original lipid dose administered. The error bars indicate the spread between two animals at each time point. As seen, the half life of clearance from the site of injection is between about 40-50 hours, determined by plotting the data on semi-log plot as shown in Figure 3 (discussed in the next section).

The accumulated level of radioactivity measured in the urine and feces is shown by the closed triangles in the figure. The total excreted radioactivity, at the

end of a 70-hour test period in this experiment, is about 72% of the original dose administered. The remaining counts are distributed among the limbs (about 5%), carcass (about 20%), and organs (about 3%). Of the roughly 3% remaining radioactivity which is localized in the organs, about 55% is found in the gut, about 34% in the liver, and less than about 5% each in the spleen, trachea, kidneys, stomach, heart, lungs, and genitals.

Example IV

Effect of Liposome Size, Dose, and Liposome Composition on Lipid Clearance From the Site of an IM Injection

The retention of tracer lipid at the sites of IM liposome injection, measured in terms of half life of clearance of the tracer from the sites, was examined as a function of liposome size, composition, and lipid dose. The clearance half lives were calculated as described in Example III, by following the loss of radioactivity in the injected limbs of male rats over a period of at least 70 hours. Each experiment generated a curve, like the closed-circle curve in Figure 2, which shows the loss of radioactivity from the sites of injection over time.

The upper dotted line in Figure 3 shows the same data expressed as a semi-log plot as a function of time. From this plot, the clearance half life of lipid from the sites of injection can be readily determined. In the particular example, the half life (at which the lipid is 50% retained) is 48 hours. The half life data, determined from the slope of the semi-log plot for each experiment, is shown in Table III below.

Liposome sizes are expressed in Table III both in terms of the pore size of the polycarbonate membrane used in sizing the MLVs, and the actual average size of

the MLVs, as determined by a Nicomp Particle Sizer (Model 200), calibrated with standardized latex beads. Thus, for example, in the first row, the MLVs injected were prepared by extrusion through a 1 micron polycarbonate membrane, but showed an average measured particle size of about 3.2 microns. The discrepancy between extruded and measured size presumable reflects liposome aggregations, which tends to occur more at higher liposome concentrations, and primarily only in composition A and E MLVs, which do not contain negatively charged lipid (PG).

The dose which is administered to the rat is measured in terms of μmole lipid per 20 or 100 μl per injection. The values shown in the dose column in the table are total doses per injection.

TABLE III

No.	Composition	Size (μM)		Dose per Injection	T-1/2
		Extruded	Measured	(μM/μl)	(days)
1	A	1.0	3.2	0.2/20	48.7
2	A	1.0	3.2	2/100	57.6
3	A	1.0	5.4	10/100	110.7
4	B	0.2	0.23	0.2/20	17.8
5	B	1.0	1.3	0.2/20	33.3
6	B	1.0	1.0	10/100	62.2
7	C	0.2	0.25	0.2/20	15.3
8	C	1.0	1.1	0.2/20	26.8
9	C	1.0	1.0	10/100	69.4
10	D	1.0	0.9	10/100	17.7

The data in rows 1-3 illustrate the effect of total lipid dose on lipid clearance from the IM site. As seen, increasing the amount of injected lipid from

about 0.2 to 10 μmole lipid produces about a twofold increase in clearance half life. The same effect is seen with Composition B MLVs (rows 5 and 6) and Composition C MLVs (rows 8 and 9).

The half life data also demonstrate that the lipid clearance rate is increased significantly with larger liposome sizes. This effect is seen from rows 4 and 5, and from rows 7 and 8, both of which compare clearance rates for MLVs extruded through 0.2 and 1.0 micron filters, respectively. The larger liposomes increased the clearance half lives by about 60-100%. The relatively high clearance rates observed with the Composition A MLVs may also reflect, at least in part, the relative large sizes of these MLVs as measured by laser particle sizing. Figure 2 shows semi-log plots from which the half lives in row 1 (dotted line) and row 4 (solid line) were calculated.

A comparison of row 1 with rows 4 and 7 (0.2/20 dose levels) and row 3 with rows 6 and 9 (10/100 dose levels) indicate a marked effect of negatively charged phospholipid (PG) on clearance rates, but little effect due to the presence of cholesterol. As suggested above, the effect of PG may be due, at least in part, to the smaller sizes observed with PG-containing liposomes. However, the measured half life for EPG MLVs (row 10) when compared with MLVs containing either 5 or 10 mole percent PG (rows 6 or 9, respectively), show a strong effect of PG concentration which does not appear to be related to liposome size.

Example V

Effect of Liposome Size, Dose, and Liposome Composition on sCT Clearance from the Site of an IM Injection

The rate of clearance of liposome-encapsulated sCT from intramuscular (IM) sites of injection was determined by procedures similar to those described in Example III, by measured the retention of ^{125}I-sCT at the sites of injection. The animals were injected with MLVs having one of the lipid compositions A, B, D, and E described in Example I, at a selected dose of 0.2, 2, or 10 μmole lipid, encapsulating about 0.5 μg of sCT, at a specific ^{125}I-Ct activity of about 1 x 10^5 cpm/0.5 μg. Free sCT was removed from the liposome suspension by centrifugation. Following injection, pairs of animals were sacrificed at period in the intervals over a 70-hour to 6-day test period, and the retention of radioactive sCT in the injected limbs of the animals, and in a number of organs, were measured as described in Example III above. Two of the injected animals were monitored for excretion of sCT by urine and feces throughout the test period.

The results of a typical experiment are shown in Figure 4, where the upper curve (circles) represents retention of ^{125}I-sCT at the sites of injection and the intermediate curve (triangles), the cumulative radioactivity excreted by the animals during the test period. As seen, between about 70-80% of the originally injected sCT is excreted after 70 hours in this experiment. The rapid clearance of F-sCT into the bloodstream is indicated by the lower curve (squares).

When the data for sCT retention are plotted in semi-log form, as shown in Figure 5, the result is a linear plot from which the clearance half life can be determined. In the example shown in the figure, the

half life is about 8 hours. The half life data determined in this manner for the four lipid compositions and the two lipid doses which were examined are given in Table IV below. In all of the studies, the MLVs had been previously sized by extrusion through 1.0 pore polycarbonate membranes, but not further extruded through smaller-pore membranes.

TABLE IV

No.	Composition	Dose (μmole/vol)	T-1/2
1	A	0.2/20	21.3
2	A	2/100	25.9
3	A	10/100	55.4
4	B	0.2/20	11.7
5	D	10/100	8.4
6	E	2/100	47.7
7	E	10/100	65.5

The data in rows 1-3 and in rows 6-7 illustrate the effect of total lipid dose on sCT clearance from the IM site. Similar to what was observed for clearance of tracer lipid, increasing the total lipid dose over a range of 0.2 to 10 μmole lipid produced a 2-3 fold increase in the clearance half life of sCT.

The data also show a marked effect of lipid composition on sCT clearance rates. The presence of PG in the MLVs (compositions B and D) resulted in much lower clearance rates than in the two non-PG-containing MLVs (compositions A and E) at equivalent doses, similar to what was found for the tracer lipid. Also, the addition of cholesterol to non-PG-containing lipids increases the sCT clearance rates noticeably. This is seen from a comparison of rows 2 and 6, which relate to

a 2/100 dose and rows 3 and 7, which relate to a 10/100 dose.

It should be noted that the half life of clearance of IM- and SQ-administered formulations are comparable. For example, formulations which have IM half lives of clearance of 20.6 hours and 9.7 hours have SQ half lives of 19.4 hours and 8.3 hours, respectively.

Table V below compares a few of the sCT clearance rates from Table IV and the corresponding rates from Table III, for the lipid tracer. Interestingly, the sCT rates are about half those of the lipid tracer, for all of the lipid composition and dose for which comparative data are available. This finding suggests that liposomes are destabilized and release their encapsulated contents predominantly at the site of injection, with lipid clearance from the injection site being handled by a different, and slower, mechanism.

TABLE V

Composition	Dose (μmole/vol)	Clearance T-1/2 sCT	Lipid
A	0.2/20	21.3	48.7
A	2/100	20.6	57.6
A	10/100	55.4	110.7
B	0.2/20	11.7	33.3
D	10/100	8.4	17.7

Example VI

Added Empty Liposome on sCT Release From Small MLVs

Reverse evaporation vesicles (REVs) containing EPC:α-T at 99:1 and encapsulated ^{125}I-sCT were prepared according to a standard REV procedure (Szoka). The vesicles were extruded through 0.4 μm and 0.2 μm polycarbonate filters, and non-encapsulated sCT was

removed from the vesicle suspension by centrifugation and washing three times in PBS. The preparation was then filtered through a sterile, 0.22 micron filter.

Empty MLVs of the same composition were prepared and extruded through a 1 μm filter as described in Example I under sterile conditions. The REVs were mixed with the MLVs in a mole ratio of 1:50 such that each IM injection contained about 1 x 10^5 cpm of ^{125}I-sCT and about 0.5 μg of sCT in 10 μmole total lipid. The mixture of liposomes were allowed to stand and aggregate overnight prior to injection. Rats were injected subcutaneously with the liposome mixture or REVs as described in Example III. The clearance of ^{125}I-radiolabel from the site of injection was followed as described in Example III, by means of a semi-log plot. The half-life data for REVs alone and REVs plus empty MLVs are shown in Table VI.

Table VI

Composition	Type of Liposome	Dose (μmole/100μl)	Half Life
A	REV	0.2	7.2
A	REV+MLV	10.0	41.9
A	MLV	10.0	55.4

As seen addition of empty MLVs to 0.2 micron REVs increased the clearance half life of CT from the REVs more than fivefold. Row 3 in the table gives the CT clearance half life for CT release from MLVs alone (as in Example IV). A comparison of rows 2 and 3 indicates that the size of the peptide-containing liposomes, as well as the size and/or amount of empty

liposomes, influences the peptide release rate from the site.

While preferred embodiments of the invention have been described herein, it will be apparent that various changes and modifications can be made without departing from the invention. In particular, although liposome delivery of CT has been used to illustrate the general principles of the invention, it will be apparent to those in the art that similar release characteristics would be observed with other liposome-impermeable compounds.

Claims

1. In administering a liposome-impermeable compound by forming a suspension of liposomes which contain the compound in entrapped form, and injecting the suspension into an intramuscular or subcutaneous site, a method for selectively increasing the rate of release of the compound from the site of injection into the bloodstream, comprising

selectively increasing the average size of liposomes injected into the site, and

selectively increasing the amount of liposome lipid injected into the site.

2. The method of claim 1, wherein the compound is a peptide selected from the group consisting of calcitonin, growth hormone, insulin, interferon, and interleukin-2.

3. The method of claim 2, wherein the hormone is calcitonin, and the hormone is encapsulated within suspension liposomes at a concentration of at least about 0.10-1.0 mg/ml.

4. The method of claim 1, which further includes reducing the amount of negatively charged phospholipid to the liposomes to increase the rate of release of the compound from the site of injection.

5. The method of claim 1, wherein the liposomes containing the entrapped compound have an average size of less than about 0.2 microns, and the average size of the injected liposomes is selectively

increased by adding increasing amount of empty liposomes having an average size greater than about 0.5 micron.

6. A liposome composition for use in administering a liposome-impermeable compound to the bloodstream from an intramuscular or subcutaneous site of injection, comprising

an aqueous suspension of liposomes containing the compound in entrapped form, and having average particles sizes less than about 0.3 microns, and

contained in the suspension, a quantity of empty liposomes, in an amount effective to prolong the half life of clearance of the compound from such injection site to a desired half life of about 1-14 days.

7. The composition of claim 6, wherein the empty liposomes have an average particle size greater than about 0.5 micron.

8. The composition of claim 6, wherein the compound is peptide selected from the group consisting of calcitonin, insulin, growth hormone, interferon, and interleukin-2.

9. The composition claim 8, wherein the hormone is calcitonin, and the concentration of hormone within the encapsulation space of the liposomes is at least about 1 mg/ml.

10. The composition of claim 6, wherein the liposomes include α-tocopherol, at a concentration of at least about 0.2 mole percent.

11. A liposome calcitonin composition comprising

a sterile, aqueous suspension of liposomes containing at least about 0.2 mole percent α-tocopherol, and

calcitonin entrapped in the liposomes at a concentration within the encapsulation space of the liposomes of at least about 1 mg/ml.

12. The composition of claim 11, wherein the liposomes contain between about 5-100 mole percent phosphatidylglycerol.

13. The composition of claim 11, wherein the liposomes have average sizes less than about 0.3 microns, and the composition further includes empty liposomes in an amount effective to increase the half life of release of calcitonin into the bloodstream, when the composition is injected into a intramuscular or subcutaneous site.

14. The composition of claim 13, wherein the empty liposomes have an average size of at least about 0.5 micron.

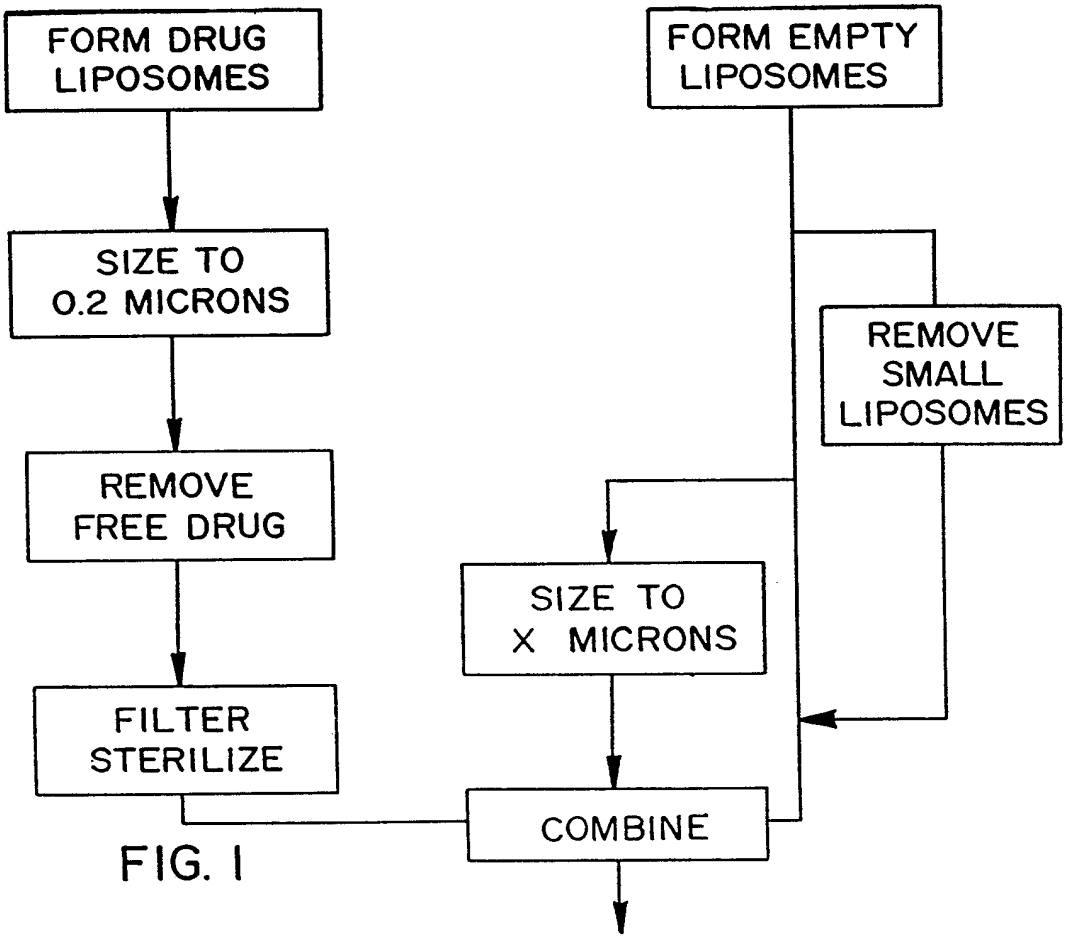

FIG. 1

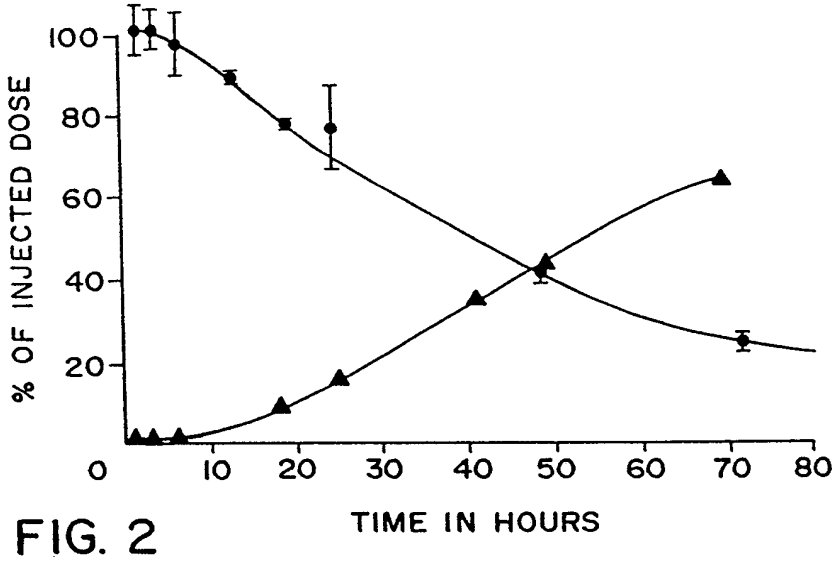

FIG. 2

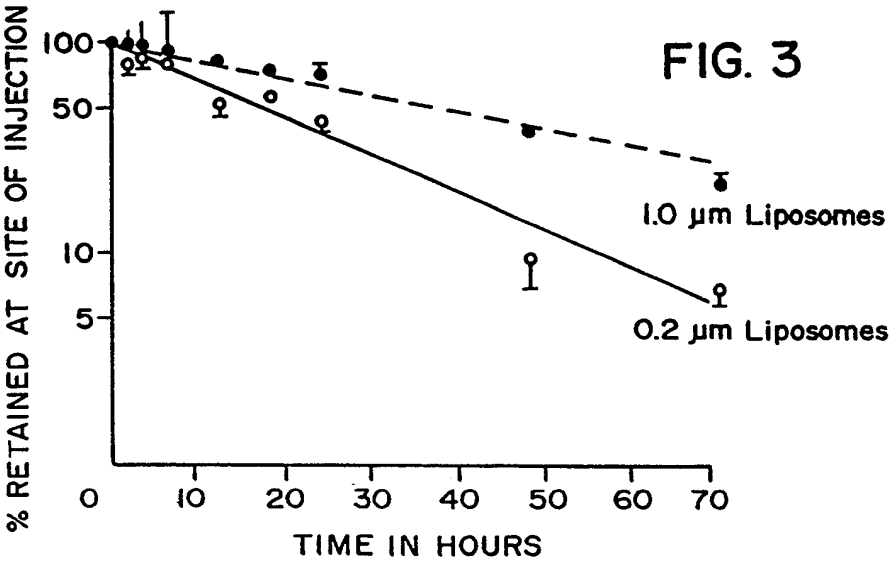

FIG. 3

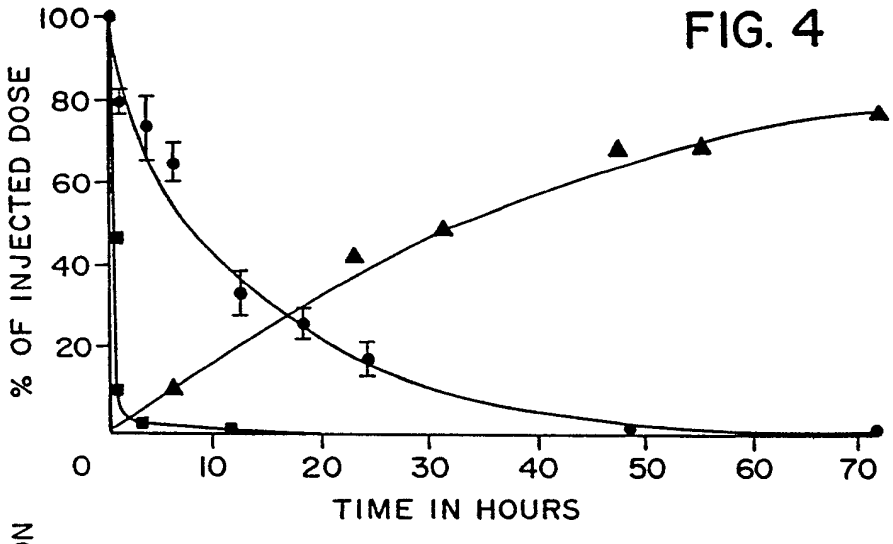

FIG. 4

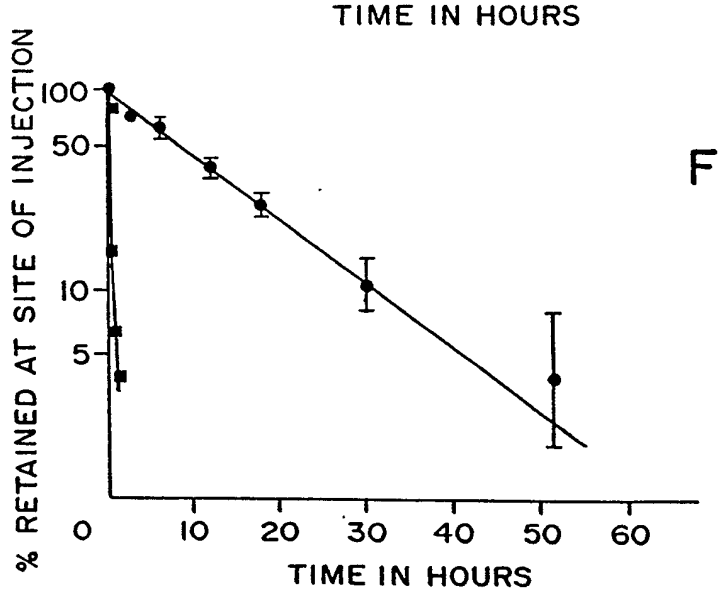

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00285

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL[4]: A01N 25/26,25/28; A61K 37/22; A61J 5/00; See Attachment
U.S. CL : 424/417,450; 264/4.1,4.3,4.6; 428/402.2

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/417,450; 264/4.1,4.3,4.6; 428/402.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US,A, 4,394,448 (SZOKA, JR. ET AL). 19 July 1983 (19.07.83), see col. 2, lines 29-30; col. 5, lines 3-7).	All
X Y	Drug Metabolism and Disposition, Volume 9, no. 6, issued 1981 (USA), Jackson, 'Intramuscular Absorption and Regional Lymphatic Uptake of Liposome-Entrapped Inulin', see the Abstract; page 535, col. 1, lines 1-6 and [illegible]-25; the Discussion.)	1 2-14
Y	Endc [illegible]logy, Volume 115, No. 2, iss [illegible]34 (USA), Fukunaga et al. 'Li [illegible] Entrapment Enhances the [illegible]alcemic Action of Par [illegible]ally Administered Cal [illegible]in', see the Abstract.	All

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
09 April 1987	16 APR 1987
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	John M. Kilcoyne John M. Kilcoyne

Form PCT/ISA/210 (second sheet) (May 1986)

Attachment to Form PCT/ISA/210, Part I.

INT. CL^4: B01J 13/02; B32B 5/16, 9/02, 9/04

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Chemical Abstracts, Volume 99, No. 7, issued 15 August 1983 (Columbus, Ohio, USA), Kang et al., 'Increasing the tumor uptake of liposomally entrapped substances by prior injection of empty liposomes', see page 305, column 1, the Abstract No. 58806c, IRCS Med. Sci.: Libr. Compend. 1983, 11(5), 420-1 (Eng).	All

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.